of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured weekly from weeks 1 to 6.

RESULTS

Expression of VCAM-1, VLA-4, and effect of Antibodies Against VCAM-1 and VLA-4 on 5TGM1 Attachment to ST2 Monolayers

Using RT-PCR, we confirmed the expression of VCAM-1 and integrin VLA-4 in bone marrow stromal cells and myeloma cells, respectively. As expected, both the ST2 stromal cell line and primary bone marrow stromal cells expressed VCAM-1, while 5TGM1 did not. In contrast, the 5TGM1 myeloma cells expressed integrin VLA-4, whereas stromal cells did not (data not shown). In addition, both anti-VCAM-1 antibody (10 ug/ml) and VLA-4 antibody (10 ug/ml) partially (50-80%) inhibited the attachment of 5TGM1 cells to ST2 monolayers, showing that VCAM-1 and the VLA-4 integrin expressed on these cells are biologically functional and that these antibodies have neutralizing activity (data not shown).

OC-like Cell Formation in the Coculture of 5TGMI Myeloma Cells with Mouse Bone Marrow Cells

On day 6 of the coculture of 5TGM1 cells and mouse marrow cells, numerous TRAP-positive multinucleated osteoclast-like (OC-like) cells were formed. These OC-like cells exhibited resorption pit formation on dentine slices, demonstrating that these cells were capable of resorbing bone, and possess an osteoclastic phenotype. In experiments using transwell inserts, formation of OC-like cells was observed when 5TGM1 cells were cultured in direct contact with bone marrow cells. In contrast, there was only a marginal number of OC-like cells formed when 5TGM1 cells were separated from marrow cells by the transwell membrane. Thus 5TGM1 cells induce osteoclast formation in mixed marrow cultures, and this induction requires direct cell-cell contact.

Effect of Antibodies Against VCAM-1 and Integrin VLA4 on OC-like Cell Formation in the Co-culture of 5TGMI and Marrow Cells

Both anti-VCAM-1 antibody (VCAM-1 Ab, 10ug/ml) and anti VLA-4-integrin antibody (alpha4beta1 Ab, 10 ug/ml) dramatically inhibited OC-like cell formation. In contrast mAb against ICAM-1, another adhesion molecule on marrow stromal cells implicated in stromal/myeloma interactions, had no effect on OC-like cell formation (Figure 1).

To determine whether this inhibition by VCAM-1 and VLA-4 mAbs was specific for 5TGM1-induced OC-like cell formation and was not due to cytotoxicity, the effects of these antibodies were examined on OC-like cell formation induced by 1,25 $(OH)_2$ D_3, a widely-used stimulator of osteoclastogenesis in mouse bone marrow cell cultures (Takahashi 1988). Neither VCAM-1 Ab nor VLA-4 mAb inhibited OC-like cell formation induced by vitamin D3, which itself had no effect on VCAM-1 expression in stromal cells (data not shown).

Effect of conditioned Medium Harvested from the Co-culture of 5TGMI and ST2 on Bone Resorption

Conditioned medium from the co-culture of 5TGM1 cells and ST2 cells showed a marked increase in bone resorption in the fetal rat long bone assay (Figure 2), while conditioned medium of 5TGM1 caused only a marginal increase, s compared to control medium. Conditioned medium from ST2 cells showed no increase in bone resorption. Thus direct cell-cell contact via VCAM-1 and VLA-4 both induces osteoclast-like cells and production of bone-resporboing factors in vitro.

Effect of Recombinant Soluble VCAM-1 (sVCAM-1) on the Production of Bone-resorbing and Osteoclastogenic Activity by 5TGMI Cells

Conditioned medium of 5TGM1 treated with a soluble recombinant form of VCAM-1 (sVCAM-1) increased bone resorption in fetal rat long bones in a dose-dependent manner, while conditioned medium obtained from untreated 5TGM1 only marginally increased bone resorption. Soluble VCAM-1 itself had no effects on bone resorption (data not shown). In the mouse marrow culture system, conditioned medium harvested from 5TGM1 cells treated with sVCAM-1 showed increased activity of OC-

like cell formation, while conditioned medium of untreated 5TGM1 exhibited only marginal activity of OC-like cell formation (Figure 3).

Expression of Rank ligand mRNA in marrow stromal cells (ST2) cultured in the presence and absence of murine myeloma cells

Because Rank ligand appears to be an important mediator of OCL formation and may be the final common pathway for the effects of osteoclastogenic cytokines on OCL formation, we have examined the expression of Rank ligand in 5TGM1 and ST2 cells both indivudually and when cocultured. We find that coculture of 5TGM1 and ST2 cells induces Rank ligand mRNA in the ST2 cells. Furthermore, while 5TGM1 cells do not expresss Rank ligand, they do so when treated with sVCAM-1 (not shown). Finally, the conditioned medium from 5TGM1 cells treated with sVCAM-1 induced Rank ligand mRNA in ST2 cells, suggesting that the VCAM-1/VLA-4 pathway produces a cytokine in myeloma cells that enhances Rank ligand expression by marrow stromal cells (data not shown).

In summary, we show that 5TGM1 cells alone produce marginal amount of activity that stimulates OC-like cell formation and bone resorption. However, when 5TGMl myeloma cells were co-cultured with bone marrow cells containing hemopoietic osteoclast precursors and stromal cells, they strongly adhered to the stromal cells and increased OC-like cell formation. There were no OC-like cells formed in the co-cultures in which 5TGM1 cells were prevented from contacting stromal cells. Furthermore, in organ cultures of fetal rat long bones the conditioned medium harvested from the co-cultures of 5TGM1 myeloma cells and ST2 bone marrow stromal cells had increased bone resorbing activity compared with conditioned medium of either ST2 or 5TGM1 alone. These data are consistent with the notion that direct cell-cell contact of 5TGMl cells with bone marrow stromal cells is required for the production of osteoclast-stimulating and bone-resorbing activity. We then determined what cell adhesion molecules were involved in the direct cell-cell interaction between 5TGM1 cells and marrow stromal cells that is necessary for the production of osteoclastogenic activity.

Our data indicate that VCAM-1 and VLA-4 integrin play a role in this cell-cell interaction, since neutralizing antibodies to these two adhesion molecules profoundly decreased OC-like cell formation in the co-cultures. The VCAM-1/VLA-4 integrin interaction is responsible for the cell-cell communication between marrow stromal cells and 5TGM1 myeloma cells leading to increased production of a osteoclastogenic and bone-resorbing activity. Finally, this bone resorbing activity in part is due to induction of Rank ligand.

Example 2: *IN VIVO* EXPERIMENTS

Our in vitro studies suggest that the interaction between VLA-4 on myeloma cells with VCAM-1 on marrow stromal cells may play a key role in the induction of bone resorbing activity by myeloma. We have taken the key step of testing this hypothesis in vivo in an animal model which accurately reflects human disease.

A. In this experiment, mice were injected with 1 e 5 5TGM1 myeloma cells, which were allowed to colonize the bone marrow. Mice were split into two groups of three, one serving as a control group, and the second treated biweekly beginning on day 8 with mAb PS/2. Levels of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured weekly from weeks 1 to 6. Treatment with mAb at a dose of 80 ug per injection (~4 mg/kg) biweekly strongly inhibited IgG2b production, indicative of significant inhibition of myeloma cell survival and growth in vivo (Figure 4). Further, the treated mice showed reduced incidence of paraplegia (all 3 untreated animals showed paraplegia on day 42, while only one of the treated animals showed paraplegia. The two treated animals with no paraplegia also showed a reduction in spleen and liver weights, which also correlate with tumor burden. Finally, the treated animals showed a reduction in tumor area by histology (from 6.71 +/- 1.74 to 0.05 +/- 0.08 square milimeters) in the tibia and femurs. There was no effect of treatment on serum calcium levels (data not shown)

B. In a parallel experiment, treatment with 40 ug PS/2 biweekly had no effect on IgG2b levels (not shown). These data show that mAb PS/2 to VLA-4 strongly inhibits the growth of established myeloma cells in a dose-dependent fashion.

C. In another in vivo experiment, 18 SCID mice were injected with 5TGM1 myeloma cells at day 0. Four mice were treated with PBS; 4 mice were treated in a – prophylactic protocol with mAb M/K-2.7 reactive against to mouse VCAM1 at a dosage of 80 ug (- 4 mg/kg) every 3 days starting at day -1 (i.e. days - 1, 2, 5, 8, and 11). In a parallel experiment using the same protocol, five mice were treated with 160 ug mAb M/K-2.7 . In addition, five mice were treated with 160 ug mAb M/K-2.7 starting at day 8 (i.e. days 8, 11, 14, 17, and 20) in a therapeutic protocol. Serum was taken from all mice on days 21, 28, and 35, and animals were X-rayed then sacrificed for histology on day 35. All three treatment groups showed a reduction in serum IgG2b levels, indicative of reduced myeloma cell burden (Figure 5). A significant effect was also observed on spleen weights at the low dose prophylactic protocol relative to control (0.23 +/- 0.14 g for control versus 0.08 +/- 0.04 for treated). In the prophylactic high dose group 4 of 5 animals showed a clear reduction in spleen weight, but the overall value was not significant because of one animal with a large spleen weight (data not presented).

D. One can investigate whether an initial high bolus dose of alpha4 integrin antagonist, followed by a maintenance dose, improves efficacy. The myeloma cells are already established in the marrow compartment, and their tight VLA-4-dependent interaction with VCAM-1 needs to be inhibited. Furthermore, presumably the greater the number of established myeloma cells, the higher the initial dose required to flush cells out into the peripheral circulation.

A larger study with the anti-VLA-4 antibody PS/2 was therefore performed. Twenty eight SCID mice were injected with 5TGM1 myeloma cells at day 0. Nine mice received no treatment; 9 mice received an isotype-matched control IgG mAb; 10 mice were treated with mAb PS/2 to alpha 4 integrin. A different therapeutic regimen

was given, in which mice were given a high dose of mAb (200 ug) on days 4,5, and 6, then a maintenance dose of 80 ug (- 4 mg/kg) every 3 days starting at day 8.

There was a statistically significant reduction in serum IgG2b when the treated group was compared to either the untreated or control IgG-treated group at weeks 3 and 4 (data not presented). Importantly, when the treated group was compared to either the untreated or control IgG-treated group there was a clear effect on survival (Figure 6).

Example 3: **OTHER IN VIVO EXPERIMENTS**

Based on the information presented herein for the first time, persons having ordinary skill in the art can readily confirm and extend the importance of the alpha4 integrins and their ligands in multiple myeloma using the murine animal model described.

The following series of experiments are well within the level of skill in the art based upon the present disclosure but serve merely to exemplify, and not limit, the types of work.

1) Dose response to mAb PS/2 to determine the optimal biweekly maintenance dose. 80 ug shows good efficacy, but 40 ug was without effect. One examines higher doses up to 20 mg/kg two or three times weekly to determine optimal dosing.
2) Patients present with disease at different stages of severity, linked to increased tumor burden. One examines the efficacy of mAb PS/2 given at different times after establishment of disease, i.e. one compares treatment inititation at 8 days (see for example Figure 4) to initiation after two, three, four and five weeks post inoculation to see how late mAb can be given to provide some relief of symptoms.
3) The effects of mAb MK-2 to murine VCAM-1 are examined, following the same parameters outlined above (dosing, timing of dosing) for mAb to VLA-4. It is anticipated that similar dosing levels will be required to see efficacy.
4) Further markers of myeloma progression are examined, including tumor burden in both marrow and extramedullary sites, quntification of bone lesions by radiographic anaysis of the skeleton by histomorphometry; measurement of rates of bone reportion by evaluation of collagen crosslinks in plasma; measurement of

monoclonal protein production in plasma; hypercalcemia where present; and mortality.

5) Multiple myeloma is currently treated inefficiently with standard chemotherapeutic regimens. The additive or synergistic effects of mAbs at optimal dosing in conjunction with, or either before or after, dosing with appropriate chemotherapeutic regimens is examined.

6) The ability of a small molecule alpha4 integrin inhibitor that is selective for one particular alpha4 integrin or is selective for several alpha4 integrins at once or the ability of combinations of such inhibitors, to mimic the effects of mAbs and block myeloma progression is examined using the protocols and outcomes described above. Small molecule inhibitors are delivered parenterally or orally, in the dosing range of 0.1 to 30 mg/kg, once or twice daily, or twice or three times weekly.

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